



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>A61K 31/70, 39/12, C12N 7/00, 15/00, C12P 21/06, C12Q 1/70, G01N 33/531</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 94/17810</b> <b>(43) International Publication Date:</b> 18 August 1994 (18.08.94)
<b>(21) International Application Number:</b> PCT/US94/02107 <b>(22) International Filing Date:</b> 10 February 1994 (10.02.94)  <b>(30) Priority Data:</b> 08/017,130      12 February 1993 (12.02.93)      US  <b>(71) Applicant:</b> THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY [US/US]; 36th and Spruce Streets, Philadelphia, PA 19104 (US).  <b>(72) Inventors:</b> PLOTKIN, Stanley, A.; 29, rue du Général-Delestraint, F-75016 Paris (FR). RICCIARDI, Robert, P.; 137 W. Forge Road, Glen Mills, PA 19342 (US). GONCZOL, Eva; Radnor House, Apartment 916, 1030 E. Lancaster Avenue, Rosemont, PA 19010 (US).  <b>(74) Agents:</b> BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).		<b>(81) Designated States:</b> CA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> RECOMBINANT CYTOMEGALOVIRUS VACCINE  <b>(57) Abstract</b>  The present invention provides a non-defective adenovirus recombinant expression system for the expression of immediate-early exon 4 proteins, said recombinant HCMV-expressing adenovirus being useful as an immunogenic composition and vaccine.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## RECOMBINANT CYTOMEGALOVIRUS VACCINE

This work was performed with government support under National Institutes of Health grants AI-07278 and  
5 HD-18957. The U.S. government has certain rights in this invention.

Field of the Invention

The present invention refers generally to a  
10 recombinant human cytomegalovirus vaccine, and more specifically to a subunit vaccine.

Background of the Invention

Cytomegalovirus (CMV) is one of a group of  
15 highly host specific herpes viruses that produce unique large cells bearing intranuclear inclusions. The envelope of the human cytomegalovirus (HCMV) is characterized by a major glycoprotein complex recently termed gB or gCI, which was previously referred to as gA.  
20 HCMV causes cytomegalic inclusion disease and has been associated with a syndrome resembling infectious mononucleosis in adults. It also induces complications in immunocompromised individuals.

CMV infection *in utero* is an important cause of  
25 central nervous system damage in newborns. Although the virus is widely distributed in the population, about 40% of women enter pregnancy without antibodies and thus are susceptible to infection. About 1% of these women undergo primary infection *in utero*. Classical  
30 cytomegalic inclusion disease is rare; however, a proportion of the infected infants, including those who were symptom free, are subsequently found to be mentally retarded.

Preliminary estimates based on surveys of  
35 approximately 4,000 newborns from several geographical areas indicate that the virus causes significant damage

of the central nervous system leading to mental deficiency in at least 10%, and perhaps as high as 25%, of infected infants. Assuming that about 1% of newborn infants per year excrete CMV and that about one fourth of those develop mental deficiency, in the United States this means approximately 10,000 brain-damaged children born per year. This is a formidable number, particularly in view of the ability of these children to survive [J. Infect. Dis., 123 (5):555 (1971)].

10 HCMV in humans has also been observed to cause serious complications and infections in the course of organ transplantations, especially with kidney and liver transplants.

Several HCMV vaccines have been developed or are in the process of development. Vaccines based on live attenuated strains of HCMV have been described. [See, e.g., S. A. Plotkin et al, Lancet, 1:528-30 (1984); S. A. Plotkin et al, J. Infect. Dis., 134:470-75 (1976); S. A. Plotkin et al, "Prevention of Cytomegalovirus Disease by Towne Strain Live Attenuated Vaccine", in Birth Defects, Original Article Series, 20(1):271-287 (1984); J. P. Glazer et al, Ann. Intern. Med., 91:676-83 (1979); and U. S. Patent 3,959,466.] A proposed HCMV vaccine using a recombinant vaccinia virus expressing HCMV glycoprotein B has also been described. [See, e.g., Cranage, M. P. et al, EMBO J., 5:3057-3063 (1986).] However, vaccinia models for vaccine delivery are believed to cause local reactions. Additionally, vaccinia vaccines are considered possible causes of encephalitis.

30 Adenoviruses have been developed previously as efficient heterologous gene expression vectors. For example, an adenovirus vector has been employed to express herpes simplex virus glycoprotein gB [D. C. Johnson et al, Virology, 164:1-14 (1988)]; human

immunodeficiency virus type 1 envelope protein [R. L. Dewar et al, J. Virol., 63:129-136 (1988)]; and hepatitis B surface antigen [A. R. Davis et al, Proc. Natl. Acad. Sci., U.S.A., 82:7560-7564 (1985); J. E. Morin et al, Proc. Natl. Acad. Sci., U.S.A., 84:4626-4630 (1987)].  
5 Adenoviruses have also been found to be non-toxic as vaccine components in humans [See, e.g., E. T. Takajui et al, J. Infect. Dis., 140:48-53 (1970); P. B. Collis et al, J. Inf. Dis., 128:74-750 (1973); and R. B. Couch et  
10 al, Am. Rev. Respir. Dis., 88:394-403 (1963)].

There remains a need in the art for additional vaccines capable of preventing CMV infection by generating neutralizing antibody and cellular responses to CMV in the human immune system.

15

#### Summary of the Invention

In one aspect, the present invention provides a non-defective recombinant adenovirus containing an immediate-early exon-4 (IE-exon-4) subunit of the HCMV  
20 free from association with any additional human proteinaceous material. In this recombinant adenovirus, the HCMV subunit is under the control of regulatory sequences capable of expressing the IE-exon 4 subunit *in vitro* and *in vivo*.

25 Another aspect of the present invention is a vaccine composition comprising a non-defective recombinant adenovirus, as described above.

In a further aspect, the invention provides a method of using the recombinant adenovirus containing the subunit gene encoding IE-exon-4, in the manufacture of a  
30 vaccine composition useful against HCMV infection. The inventors have found that presenting these HCMV subunit proteins expressed by *in vivo* transcription of the gene to a vaccinate is particularly capable of eliciting a  
35 protective immune response.

In yet a further aspect the invention provides an adenovirus-produced HCMV IE-exon-4 subunit, which subunit may also form vaccine compositions to protect humans against HCMV.

5 In still a further aspect, the present invention provides a novel murine model useful for demonstrating cytotoxic T lymphocyte (CTL) response to individual HCMV proteins.

10 Other aspects and advantages of the present invention are described further in the following detailed description of preferred embodiments of the present invention.

#### Detailed Description of the Invention

15 The present invention provides novel immunogens and vaccine components for HCMV which comprise an adenovirus expression system capable of expressing a selected HCMV subunit gene *in vivo*. Alternatively the selected subunit for use in an immunogenic composition, 20 *i.e.*, a composition which elicits a humoral and/or a cell-mediated immune response, may be expressed in, and isolated from, the recombinant adenovirus expression system. Such an immunogenic composition may be used in a vaccine for protecting against human CMV infection.

25 As provided by the present invention, any adenovirus strain capable of replicating in mammalian cells *in vitro* may be used to construct an expression vector for the selected HCMV subunit. However, a preferred expression system involves a non-defective 30 adenovirus strain, including, but not limited to, adenovirus type 5. Alternatively, other desirable adenovirus strains may be employed which are capable of being orally administered, for use in expressing the CMV subunit *in vivo*. Such strains useful for *in vivo* 35 production of the subunit in addition to adenovirus-5

strains include adenovirus type 4, 7, and 21 strains. [See, e.g., Takajui et al, cited above]. Appropriate strains of adenovirus, including those identified above and those employed in the examples below are publicly  
5 available from sources such as the American Type Culture Collection, Rockville, Maryland.

The presently preferred subunit protein for use in the present invention is the HCMV IE-exon 4 subunit. The full length IE1 gene was reported by Stenberg et al,  
10 J. Virol., 49:190-199 (1984). An XbaI E fragment containing the exon 4 subunit of the IE (or IE1) gene of the Towne strain of HCMV was reported to GenBank, Los Alamos, New Mexico in September 15, 1989 by Stenberg et al. The nucleic acid sequences of the coding region of  
15 the IE-exon-4 are provided in SEQ ID NO:1, in which the native TC nucleotides which precede the lysine codon have been modified to the ATG initiation codon. SEQ ID NO:2 provides amino acid sequences of the IE-exon 4 protein.

In the practice of one embodiment of this  
20 invention the HCMV IE-exon 4 subunit may be produced *in vitro* by recombinant techniques in large quantities sufficient for use in a subunit vaccine. Alternatively, more than one HCMV subunit may be employed in a vaccine according to the teachings of the present invention.

25 Alternatively, the recombinant adenovirus containing the subunit may itself be employed as an immunogen or vaccine component, capable of expressing the subunit *in vivo*. One embodiment of the invention provides a replication competent adenovirus-5 vector  
30 carrying the HCMV IE-exon 4 gene.

The desired subunit may be isolated from an available strain of HCMV for insertion into the selected adenovirus. A number of strains of human CMV have been isolated. For example, the Towne strain of CMV, a  
35 preferred strain for use in preparation of a vaccine of

this invention because of its broad antigenic spectrum and its attenuation, was isolated from the urine of a two month old male infant with cytomegalic inclusion disease (symptoms - central nervous system damage and  
5 hepatosplenomegaly). This strain of CMV was isolated by Stanley A. Plotkin, M.D. of The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania, and is described in J. Virol., 11 (6): 991 (1973). This strain is freely available from The Wistar Institute or from the  
10 American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, USA, under accession number VR-977. However, other strains of CMV useful in the practice of this invention may be obtained from depositories like the ATCC or from other institutes or  
15 universities.

In addition to isolating the desired IE-exon 4 subunit from an available strain of HCMV for insertion into the selected adenovirus, the subunit sequence can be chemically synthesized by resort to conventional methods  
20 known to one of skill in the art and, e.g., SEQ ID NOS: 1 and 2. Alternatively, the sequence may be purchased from commercial sources.

The recombinant adenovirus of the present invention may also contain multiple copies of the HCMV  
25 subunit. Alternatively, the recombinant virus may contain more than one HCMV subunit type, so that the virus may express two or more HCMV subunits or immediate early antigens and subunits together. The sequences of other HCMV subunits of two HCMV strains have been  
30 published [See, e.g., Mach et al, J. Gen. Virol., 67:1461-1467 (1986); Cranage et al, (1986) cited above; and Spaete et al, Virol., 167:207-225 (1987)].

In the construction of the adenovirus vector of the present invention, the CMV subunit sequence is  
35 preferably inserted in an adenovirus strain under the



control of an expression control sequence in the virus itself. The adenovirus vector of the present invention preferably contains other sequences of interest in addition to the HCMV subunit. Such sequences may include  
5 regulatory sequences, enhancers, suitable promoters, secretory signal sequences and the like. The techniques employed to insert the subunit sequence into the adenovirus vector and make other alterations in the viral DNA, e.g., to insert linker sequences and the like, are  
10 known to one of skill in the art. See, e.g., T. Maniatis et al, "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). Thus, given the disclosures contained herein the construction of suitable adenovirus expression vectors  
15 for expression of an HCMV IE-exon 4 subunit protein is within the skill of the art. Example 1 below describes in detail the construction of a non-defective adenovirus containing the HCMV IE-exon-4 subunit.

The recombinant adenovirus itself, constructed  
20 as described above, may be used directly as an immunogen or a vaccine component. According to this embodiment of the invention, the recombinant adenovirus, containing the HCMV subunit, e.g., the IE-exon-4 subunit, is introduced directly into the patient by vaccination. The  
25 recombinant virus, when introduced into a patient directly, infects the patient's cells and produces the CMV subunit in the patient's cells. The inventors have found that this method of presenting these HCMV genes to a vaccinee is particularly capable of eliciting a  
30 protective immune response. Examples 2 and 3 below demonstrate the ability of the adenovirus recombinant of this invention, Ad-IE, containing subunit IE-exon-4 to elicit a CTL response from immunized mice.

According to another embodiment of this  
35 invention, once the recombinant viral vector containing

the CMV subunit protein, e.g., the IE-exon 4 subunit, is constructed, it may be infected into a suitable host cell for *in vitro* expression. The infection of the recombinant viral vector is performed in a conventional manner. [See, Maniatis et al, supra.] Suitable host cells include mammalian cells or cell lines, e.g., A549 (human lung carcinoma) or 293 (transformed human embryonic kidney) cells.

The host cell, once infected with the recombinant virus of the present invention, is then cultured in a suitable medium, such as Minimal Essential Medium (MEM) for mammalian cells. The culture conditions are conventional for the host cell and allow the subunit, e.g., IE-exon4, to be produced either intracellularly, or secreted extracellularly into the medium. Conventional protein isolation techniques are employed to isolate the expressed subunit from the selected host cell or medium.

When expressed *in vitro* and isolated from culture, the subunit, e.g., IE-exon4, may then be formulated into an appropriate vaccine composition. Such compositions may generally contain one or more of the recombinant CMV subunits.

The preparation of a pharmaceutically acceptable vaccine composition, having appropriate pH, isotonicity, stability and other conventional characteristics is within the skill of the art. Thus, such vaccines may optionally contain other components, such as adjuvants and/or carriers, e.g., aqueous suspensions of aluminum and magnesium hydroxides.

Thus, the present invention also includes a method of vaccinating humans against human CMV infection with the recombinant adenovirus vaccine composition. This vaccine composition is preferably orally administered, because adenoviruses are known to replicate in cells of the stomach. Previous studies with

adenoviruses have shown them to be safe when administered orally [see, e.g., Collis et al, cited above]. However, the present invention is not limited by the route of administration selected for the vaccine.

5           When the recombinant adenovirus is administered as the vaccine, a dosage of between  $10^5$  and  $10^8$  plaque forming units may be used. Additional doses of the vaccines of this invention may also be administered where considered desirable by the physician. The dosage  
10 regimen involved in the method for vaccination against CMV infection with the recombinant virus of the present invention can be determined considering various clinical and environmental factors known to affect vaccine administration.

15           Alternatively, the vaccine composition may comprise one or more recombinantly-produced human CMV subunit proteins, preferably including the IE-exon-4 subunit. The *in vitro* produced subunit proteins may be introduced into the patient in a vaccine composition as  
20 described above, preferably employing the oral, nasal or subcutaneous routes of administration. The presence of the subunit produced either *in vivo* or as part of an *in vitro* expressed subunit administered with a carrier, stimulates an immune response in the patient. Such an  
25 immune response is capable of providing protection against exposure to the whole human CMV microorganism. The dosage for all routes of administration of the *in vitro* vaccine containing one or more of the CMV subunit proteins is generally greater than 20 micrograms of  
30 protein per kg of patient body weight, and preferably between 40 and 80 micrograms of protein per kilogram.

          The utility of the recombinant adenoviruses of the present invention is demonstrated through the use of a novel mouse experimental model which characterizes  
35 cytotoxic T lymphocyte (CTL) responses to individual

proteins of strictly human-restricted viruses. For example, the model as used herein is based on the use of two types of recombinant viruses, an adenovirus and a canarypox virus, both expressing a gene of the same HCMV protein. This model is useful in identifying immunodominant HCMV proteins and immunodominant epitopes of individual proteins to incorporate into an appropriate immunizing vector, analysis of proteins of various HCMV strains, immunization protocols and the longevity of cell-mediated immunity to individual proteins or epitopes; and investigation of the optimal vector for effective introduction of a certain antigen or epitope to the host immune system.

According to this model, mice are immunized with one recombinant, such as that of the invention, and CTL activity tested in target cells infected with the other recombinant. Specifically, Example 2 below provides a murine model of the cytotoxic T lymphocyte (CTL) response to the glycoprotein B (gB) gene of human cytomegalovirus (HCMV) based on the use of gB-expressing adenovirus (Ad-gB) and several poxvirus recombinants.

The following examples illustrate the construction of a non-defective adenovirus strain capable of expressing the HCMV IE-exon-4, and the efficacy of this composition as an HCMV vaccine. These examples are illustrative only and do not limit the scope of the present invention.

#### Example 1 - Construction of Ad-IE exon-4 recombinant virus

The protection of humans from CMV infection or virus-induced diseases is based on antibody dependent and/or T-cell dependent immune responses. The following experimental data demonstrates that an adenovirus recombinant containing the major immediate early (IE)

gene of HCMV elicits a protective immune response in mice. The nucleic acid sequences of the coding region of the IE-exon-4 are provided in SEQ ID NO:1, in which the native TC nucleotides which precede the lysine codon have been modified to the ATG initiation codon.

To construct the IE-exon-4 adenovirus recombinant, the polymerase chain reaction (PCR) technique was used to amplify the exon 4 portion of the IE gene from purified HCMV genomic DNA (Towne strain). The PCR primers were synthesized so as to incorporate the proper restriction endonuclease cleavage site, XbaI, (underlined in SEQ ID NOS: 3 and 4 below) for insertion into the XbaI site of the adenovirus vector. In addition, the 5' primer was also modified so that an ATG start translation codon was inserted at the first amino acid position of exon 4. The oligonucleotides used as primers were the following:

5' IE-exon 4: SEQ ID NO:3:

5'-TTATCCTCC TCTAGA ATGAAACAGATTAAG

3' IE-exon 4: SEQ ID NO:4:

5'-ATATATATAT TCTAGA GTTTACTGGTCGAC

The 5' oligonucleotide corresponds to nucleotide positions 1 to 27 (sense orientation) and the 3' oligonucleotide corresponds to nucleotide positions 1251 to 1222 (anti-sense orientation) of an XbaI E fragment of the HCMV IE1 gene (Towne strain) available from GenBank, Los Alamos, New Mexico (Accession #M11630, Code #8SMIE4). This fragment was used as an Exon 4 gene template for the PCR reaction. The full length IE1 gene was reported by Stenberg et al, J. Virol., 49:190-199 (1984).

In order to clone the IE1 exon 4, the 5' and 3' primers (400 ng each) were mixed with 0.1 µg of purified HCMV genomic DNA and the DNA was amplified using the Perkin-Elmer amplitaq kit. The final reaction volume was 100 µl and the thermocycling conditions were 94°C, 1 min;

52°C, 1 min; 72°C, 1 min, repeated for a total of 35 cycles. Amplified DNA was purified by cutting the proper size DNA fragment out of a 1.2% agarose gel, digested with XbaI, repurified by cutting the digested fragments out of a 1.2% agarose gel and then ligated into the XbaI site of the cloning vector pAd-5. Positive recombinants were verified by DNA sequence analysis. Sequence analysis confirmed the orientation of the clones since the XbaI digested DNA fragments could be inserted into the adenovirus vector in two different orientations.

In this construct the E3 coding region (between map units 78.5 and 84.0) of the adenovirus is replaced by the exon-4 fragment. The correct orientation allows for the proper transcription of the gene fragment (in the sense orientation) from the adenovirus E3 promoter.

The exon-4 product of the HCMV-IE gene was shown to be a target for CD8 cytotoxic and CD4 lymphoproliferative T cell responses in humans. The Ad-IE-exon-4 construct is non-defective in replication (i.e., capable of replicating normally) in tissue culture cells.

This Ad-IE-exon-4 recombinant was used in the *in vitro* cytotoxic T lymphocyte (CTL) assay and mouse model described below.

#### Example 2 - CTL Assay-Murine Model

This CTL assay is a system in which two types of viral expression vectors, poxvirus and adenovirus, carrying the same HCMV IE-exon 4 subunit gene, are alternately used for immunization of animal or for infection of target cells to show that HCMV IE-exon 4 subunit is an inducer of CTL in mice. Using this model system, the relative immunogenicities of both a gB antigen expressed by different recombinant viruses and the IE exon 4 subunit antigen has been evaluated.

#### A. Recombinant Viruses Used in CTL Assays

The following recombinant viruses were used in the CTL assay of Example 3 below to demonstrate the vaccine utility of the recombinant adenoviruses of the present invention.

Wild-type human adenovirus type 5 (WT-Ad) and the non-defective adenovirus-gB recombinant (Ad-gB) (prepared as described in European Patent Publication No. 389,286 (Sept. 26, 1990) and G. S. Marshall et al, J. Infect. Dis., 162:1177-1181 (1990)) were propagated in human lung carcinoma A549 cells [ATCC CCL185], using standard procedures.

An E3-deleted adenovirus type 5 mutant lacking the XbaI D fragment of adenovirus DNA (Ad5ΔE3) was constructed by overlap recombination, using plasmid pAd-5 mu 59.5-100, which was deleted in E3 sequences (mu 78.5-84) using the techniques described in EP No. 389,286 and Marshall et al, cited above, and pAd-5 mu 0-75.9.

A vaccinia virus recombinant containing the gB subunits (VacC-gB) described previously in Gonczol et al, Vaccine, 9:631-637 (1991) and the parental Copenhagen strain of vaccinia, VC-2 (also known as wild-type vaccinia (WT-Vac)) were grown in Vero cells [E. Gonczol et al, Vaccine, 8:130-136 (1990); J. Tartaglia et al, Crit. Rev. Immunol., 10:13-30 (1990)].

The vaccinia WR strain [obtained from Dr. Enzo Paoletti, Virogenetics Corp, Troy, NY] was used to develop a recombinant expressing HCMV-gB ((VacW)-gB). This recombinant was derived using a strategy similar to that described for the VacC-gB recombinant (Gonczol et al., cited above).

A canarypox recombinant [ALVAC-CMV (vCP139) which is subsequently referred to as Cp-gB] expressing the HCMV-gB gene was constructed using a strategy similar to that described for a canarypox-rabies recombinant in

Taylor et al., Vaccine, 9:190-193 (1991) [also obtained from Dr. Enzo Paoletti]. Briefly, the gene encoding the HCMV (Towne strain) glycoprotein B was inserted into a canarypox donor plasmid consisting of a polylinker  
5 flanked by genomic sequence from which a nonessential gene was specifically deleted (at a unique EcoRI site within a 3.3 kbp PvuII subgenomic fragment of canarypox DNA). Expression of the gB protein gene was placed under the transcriptional control of an early/late vaccinia  
10 virus promoter (H6) previously described [Percus et al., J. Virol., 63:3829-3835 (1989)]. Cp-gB was derived and plaque-purified by standard methods [Panicali and Paoletti, Proc. Natl. Acad. Sci. USA, 79:4927-4931 (1982)]. The Cp-gB recombinant and parental canarypox  
15 virus (WT-Cp) were propagated on primary chick embryo fibroblasts (CEF) cells [ATCC CRL 1590].

B. CTL Response of Ad-gB Recombinant

For immunization of mice, Ad-gB and WT-Ad were purified by CsCl gradient centrifugation. VacC-gB,  
20 VacW-gB and WT-Vac were purified by sucrose gradient centrifugation, and Cp-gB and WT-Cp were concentrated on sucrose cushion.

Six- to 8-week-old female BALB/c and CBA mice (from Harlan Sprague-Dawley and Jackson) and 12-week-old  
25 male BALB/k mice (from The Wistar Institute Animal Facility) were immunized intraperitoneally (i.p.) with the recombinant viruses described above at  $1-5 \times 10^8$  pfu unless otherwise stated.

One to 12 weeks later, spleens were aseptically  
30 removed and cell suspensions were prepared by gently pressing the spleens through a stainless steel mesh. Cells were suspended at  $2.5 \times 10^6$  viable cells/ml in RPMI 1640 medium containing 5% FBS (Gibco),  $2 \times 10^{-5}$  M 2-mercaptoethanol, 14 mM HEPES buffer, glutamine and 50  
35  $\mu$ g/ml gentamicin. Spleen cell cultures were restimulated



15

in vitro with Ad-gB (multiplicity of infection (m.o.i.) = 10) or Vacc-gB (m.o.i. = 0.5 ) infected autologous spleen cells for 5 days in 24-well plates. Cytolytic activity of nonadherent spleen cells was tested in a chromium release assay which was performed as follows.

(1) T-cell subset depletion

For in vitro depletion of CD4 or CD8 cells, 3 x 10<sup>6</sup> spleen cells were incubated with anti-mouse CD4 monoclonal antibody (MAb) [Pharmingen; Cat.3:01061 D; 20 µg/3x10<sup>6</sup> cells] or CD8 MAb [Accurate; Cat. #:CL-8921; diluted 1:4] for 60 minutes at 4°C, and further incubated in the presence of rabbit complement [Accurate; Low-tox M; diluted 1:10] for 30 minutes at 37°C. The cells were washed twice and used as effector cells in a <sup>51</sup>Cr-release test.

(2) Chromium release assay

P815 (H-2<sup>d</sup>) [ATCC TIB 64], mouse MC57 (H-2<sup>b</sup>) cells [also termed MC-57G, D.P. Aden et al, Immunogenetics, 3:209-221 (1976)] and mouse NCTC clone 929 (H-2<sup>k</sup>) cells [ATCC CCL 1] were used as target cells. The HCMV neutralization titer of mouse sera was determined on MRC-5 cells [ATCC CCL 171] by the microneutralization method as described in Gonczol et al., J. Virol. Methods, 14:37-41 (1986).

The target cells were infected with Ad-gB or Ad-5ΔE3 (multiplicity of infection (m.o.i.) = 40-80, 40 hours) or with Vacc-gB or WT-Vacc (m.o.i. = 5-10, 4 hours). Target cells were washed in the modified RPMI 1640 medium described above and 2 x 10<sup>6</sup> cells were labeled with 100 µCi of [<sup>51</sup>Cr]NaCrO<sub>4</sub> [Amersham, specific activity 250-500 mCi/mg] for 1 hour. The labeled target cells were washed 3 times in phosphate-buffered saline (PBS) and then mixed with the effector cells at various effector:target ratios in triplicate using 96-well U-bottomed microtiter plates and incubated for 4 hours.

16

Percentage specific  $^{51}\text{Cr}$  release was calculated as:  $[(\text{cpm experimental release} - \text{cpm spontaneous release}) / (\text{cpm maximal release} - \text{cpm spontaneous release})] \times 100$ . Standard deviation of the mean of triplicate cultures was less than 10%, and spontaneous release was always less than 25%.

C. CTL Response of Ad-IE exon-4 recombinant

The CTL-assay was carried out as described above for gB. In this CTL assay mice were immunized with Ad-IE-exon-4 recombinant virus and target cells were infected with Vac(WR strain)-IE recombinant virus or parental vaccinia virus. Briefly, mice were immunized i.p. with Ad-IE-exon-4 at  $1-2 \times 10^8$  plaque forming units (p.f.u). These spleen cell cultures were restimulated in vitro with Ad-IE-exon-4 or Vaccinia (Copenhagen strain)-IE-exon-4 (Vac-IE-exon-4)-infected autologous spleen cells for 5 days. Cytolytic activity of non-adherent spleen cells was tested in a chromium release assay. The vaccinia recombinants were provided by Dr. Paoletti, Virogenetics Corporation, Troy, New York.

For the chromium release assay, MHC class-I matched and mismatched target cells were infected with Ad-IE-exon-4, or parental adenovirus, or with Vac-IE-exon-4 or parental vaccinia virus. Percentage specific  $^{51}\text{chromium}$  release was calculated as:  $[(\text{cpm experimental release} - \text{cpm spontaneous release}) / (\text{cpm maximal release} - \text{cpm spontaneous release})] \times 100$ .

When tested in the CTL assay described above, the CBA mice immunized with the Ad-IE-exon-4 recombinant developed a HCMV-IE-exon-4 specific cytotoxic T cell response.

Example 3 - Protection Study using Ad-IE exon-4

HCMV-protein-specific protection was demonstrated in Ad-HCMV immunized mice from a vaccinia-

HCMV recombinant-induced encephalitis/meningitis and death, as follows. The model is described above.

In this experiment, CBA mice were immunized i.p. with  $2 \times 10^8$  p.f.u. of the Ad-HCMV subunit protein recombinant virus, e.g. Ad-IE-exon 4 of Example 1, and 5-18 days later were challenged intracerebrally (i.c.) with a lethal dose of a vaccinia(WR strain)-HCMV recombinant virus (e.g. Vac(WR)-gB). Vaccinia(WR strain)-IE or vaccinia(WR strain)-gB recombinant viruses were obtained from Dr. Paoletti, Virogenetics Corporation, Troy, NY. The WR-strain of vaccinia is neurovirulent for mice.

When tested in this mouse model, CBA mice immunized with the Ad-IE-exon-4 recombinant were protected against a lethal dose of vaccinia WR-IE recombinant virus. The protection was HCMV-IE protein specific. Ninety percent of CBA mice, immunized i.p. with Ad-IE-exon-4 recombinant virus were protected against a lethal dose of Vac(WR-strain)-IE recombinant virus, inoculated intracerebrally. Control mice, immunized with Ad-gB recombinant virus or parental adenovirus and challenged later with the Vac(WR)-IE recombinant, died within 7 days after challenge, demonstrating that protection was IE-exon-4 protein specific.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. For example, use of other appropriate non-defective adenovirus strains for construction of analogous expression systems to express the HCMV IE-exon-4 gene may be constructed according to the disclosure of the present invention. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: The Wistar Institute of Anatomy and  
Biology

(ii) TITLE OF INVENTION: Recombinant Cytomegalovirus  
Vaccine

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Howson and Howson
- (B) STREET: Spring House Corporate Cntr,  
PO Box 457
- (C) CITY: Spring House
- (D) STATE: Pennsylvania
- (E) COUNTRY: USA
- (F) ZIP: 19477

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0,  
Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/017,130
- (B) FILING DATE: 12-FEB-1993

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Bak, Mary E.
- (B) REGISTRATION NUMBER: 31,215
- (C) REFERENCE/DOCKET NUMBER: WST6BPCT

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 215-540-9200
- (B) TELEFAX: 215-540-5818

19

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1221 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1218

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG AAA CAG ATT AAG GTT CGA GTG GAC ATG CTG CGG CAT	39
Met Lys Gln Ile Lys Val Arg Val Asp Met Leu Arg His	
1 5 10	
AGA ATC AAG GAG CAC ATG CTG AAA AAA TAT ACC CAG ACG	78
Arg Ile Lys Glu His Met Leu Lys Lys Tyr Thr Gln Thr	
15 20 25	
GAA GAG AAA TTC ACT GGC GCC TTT AAT ATG ATG GGA GGA	117
Glu Glu Lys Phe Thr Gly Ala Phe Asn Met Met Gly Gly	
30 35	
TGT TTG CAG AAT GCC TTA GAT ATC TTA GAT AAG GTT CAT	156
Cys Leu Gln Asn Ala Leu Asp Ile Leu Asp Lys Val His	
40 45 50	
GAG CCT TTC GAG GAG ATG AAG TGT ATT GGG CTA ACT ATG	195
Glu Pro Phe Glu Glu Met Lys Cys Ile Gly Leu Thr Met	
55 60 65	
CAG AGC ATG TAT GAG AAC TAC ATT GTA CCT GAG GAT AAG	234
Gln Ser Met Tyr Glu Asn Tyr Ile Val Pro Glu Asp Lys	
70 75	
CGG GAG ATG TGG ATG GCT TGT ATT AAG GAG CTG CAT GAT	273
Arg Glu Met Trp Met Ala Cys Ile Lys Glu Leu His Asp	
80 85 90	
GTG AGC AAG GGC GCC GCT AAC AAG TTG GGG GGT GCA CTG	312
Val Ser Lys Gly Ala Ala Asn Lys Leu Gly Gly Ala Leu	
95 100	
CAG GCT AAG GCC CGT GCT AAA AAG GAT GAA CTT AGG AGA	351
Gln Ala Lys Ala Arg Ala Lys Lys Asp Glu Leu Arg Arg	
105 110 115	

20

AAG ATG ATG TAT ATG TGC TAC AGG AAT ATA GAG TTC TTT	390
Lys Met Met Tyr Met Cys Tyr Arg Asn Ile Glu Phe Phe	
120 125 130	
ACC AAG AAC TCA GCC TTC CCT AAG ACC ACC AAT GGC TGC	429
Thr Lys Asn Ser Ala Phe Pro Lys Thr Thr Asn Gly Cys	
135 140	
AGT CAG GCC ATG GCG GCA TTG CAG AAC TTG CCT CAG TGC	468
Ser Gln Ala Met Ala Ala Leu Gln Asn Leu Pro Gln Cys	
145 150 155	
TCC CCT GAT GAG ATT ATG GCT TAT GCC CAG AAA ATA TTT	507
Ser Pro Asp Glu Ile Met Ala Tyr Ala Gln Lys Ile Phe	
160 165	
AAG ATT TTG GAT GAG GAG AGA GAC AAG GTG CTC ACG CAC	546
Lys Ile Leu Asp Glu Glu Arg Asp Lys Val Leu Thr His	
170 175 180	
ATT GAT CAC ATA TTT ATG GAT ATC CTC ACT ACA TGT GTG	585
Ile Asp His Ile Phe Met Asp Ile Leu Thr Thr Cys Val	
185 190 195	
GAA ACA ATG TGT AAT GAG TAC AAG GTC ACT AGT GAC GCT	624
Glu Thr Met Cys Asn Glu Tyr Lys Val Thr Ser Asp Ala	
200 205	
TGT ATG ATG ACC ATG TAC GGG GGC ATC TCT CTC TTA AGT	663
Cys Met Met Thr Met Tyr Gly Gly Ile Ser Leu Leu Ser	
210 215 220	
GAG TTC TGT CGG GTG CTG TCC TGC TAT GTC TTA GAG GAG	702
Glu Phe Cys Arg Val Leu Ser Cys Tyr Val Leu Glu Glu	
225 230	
ACT AGT GTG ATG CTG GCC AAG CGG CCT CTG ATA ACC AAG	741
Thr Ser Val Met Leu Ala Lys Arg Pro Leu Ile Thr Lys	
235 240 245	
CCT GAG GTT ATC AGT GTA ATG AAG CGC CGC ATT GAG GAG	780
Pro Glu Val Ile Ser Val Met Lys Arg Arg Ile Glu Glu	
250 255 260	
ATC TGC ATG AAG GTC TTT GCC CAG TAC ATT CTG GGG GCC	819
Ile Cys Met Lys Val Phe Ala Gln Tyr Ile Leu Gly Ala	
265 270	
GAT CCT CTG AGA GTC TGC TCT CCT AGT GTG GAT GAC CTA	858
Asp Pro Leu Arg Val Cys Ser Pro Ser Val Asp Asp Leu	
275 280 285	

21

CGG GCC ATC GCC GAG GAG TCA GAT GAG GAA GAG GCT ATT	897
Arg Ala Ile Ala Glu Glu Ser Asp Glu Glu Ala Ile	
290 295	
GTA GCC TAC ACT TTG GCC ACC CGT GGT GCC AGC TCC TCT	936
Val Ala Tyr Thr Leu Ala Thr Arg Gly Ala Ser Ser	
300 305 310	
GAT TCT CTG GTG TCA CCC CCA GAG TCC CCT GTA CCC GCG	975
Asp Ser Leu Val Ser Pro Pro Glu Ser Pro Val Pro Ala	
315 320 325	
ACT ATC CCT CTG TCC TCA GTA ATT GTG GCT GAG AAC AGT	1014
Thr Ile Pro Leu Ser Ser Val Ile Val Ala Glu Asn Ser	
330 335	
GAT CAG GAA GAA AGT GAG CAG AGT GAT GAG GAA GAG GAG	1053
Asp Gln Glu Glu Ser Glu Gln Ser Asp Glu Glu Glu Glu	
340 345 350	
GAG GGT GCT CAG GAG GAG CGG GAG GAC ACT GTG TCT GTC	1092
Glu Gly Ala Gln Glu Glu Arg Glu Asp Thr Val Ser Val	
355 360	
AAG TCT GAG CCA GTG TCT GAG ATA GAG GAA GTT GCC CCA	1131
Lys Ser Glu Pro Val Ser Glu Ile Glu Glu Val Ala Pro	
365 370 375	
GAG GAA GAG GAG GAT GGT GCT GAG GAA CCC ACC GCC TCT	1170
Glu Glu Glu Glu Asp Gly Ala Glu Glu Pro Thr Ala Ser	
380 385 390	
GGA GGC AAG AGC ACC CAC CCT ATG GTG ACT AGA AGC AAG	1209
Gly Gly Lys Ser Thr His Pro Met Val Thr Arg Ser Lys	
395 400	
GCT GAC CAG TAA	1221
Ala Asp Gln	
405	

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 406 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

Met	Lys	Gln	Ile	Lys	Val	Arg	Val	Asp	Met	Leu	Arg	His	Arg
1				5					10				
Ile	Lys	Glu	His	Met	Leu	Lys	Lys	Tyr	Thr	Gln	Thr	Glu	Glu
15					20					25			
Lys	Phe	Thr	Gly	Ala	Phe	Asn	Met	Met	Gly	Gly	Cys	Leu	Gln
	30					35					40		
Asn	Ala	Leu	Asp	Ile	Leu	Asp	Lys	Val	His	Glu	Pro	Phe	Glu
		45					50					55	
Glu	Met	Lys	Cys	Ile	Gly	Leu	Thr	Met	Gln	Ser	Met	Tyr	Glu
			60					65					70
Asn	Tyr	Ile	Val	Pro	Glu	Asp	Lys	Arg	Glu	Met	Trp	Met	Ala
				75						80			
Cys	Ile	Lys	Glu	Leu	His	Asp	Val	Ser	Lys	Gly	Ala	Ala	Asn
85					90					95			
Lys	Leu	Gly	Gly	Ala	Leu	Gln	Ala	Lys	Ala	Arg	Ala	Lys	Lys
	100					105					110		
Asp	Glu	Leu	Arg	Arg	Lys	Met	Met	Tyr	Met	Cys	Tyr	Arg	Asn
		115					120					125	
Ile	Glu	Phe	Phe	Thr	Lys	Asn	Ser	Ala	Phe	Pro	Lys	Thr	Thr
			130					135					140
Asn	Gly	Cys	Ser	Gln	Ala	Met	Ala	Ala	Leu	Gln	Asn	Leu	Pro
				145					150				
Gln	Cys	Ser	Pro	Asp	Glu	Ile	Met	Ala	Tyr	Ala	Gln	Lys	Ile
155					160					165			
Phe	Lys	Ile	Leu	Asp	Glu	Glu	Arg	Asp	Lys	Val	Leu	Thr	His
	170					175					180		
Ile	Asp	His	Ile	Phe	Met	Asp	Ile	Leu	Thr	Thr	Cys	Val	Glu
		185					190					195	
Thr	Met	Cys	Asn	Glu	Tyr	Lys	Val	Thr	Ser	Asp	Ala	Cys	Met
			200					205					210
Met	Thr	Met	Tyr	Gly	Gly	Ile	Ser	Leu	Leu	Ser	Glu	Phe	Cys
				215					220				



23

Arg Val Leu Ser Cys Tyr Val Leu Glu Glu Thr Ser Val Met  
 225 230 235  
 Leu Ala Lys Arg Pro Leu Ile Thr Lys Pro Glu Val Ile Ser  
 240 245 250  
 Val Met Lys Arg Arg Ile Glu Glu Ile Cys Met Lys Val Phe  
 255 260 265  
 Ala Gln Tyr Ile Leu Gly Ala Asp Pro Leu Arg Val Cys Ser  
 270 275 280  
 Pro Ser Val Asp Asp Leu Arg Ala Ile Ala Glu Glu Ser Asp  
 285 290  
 Glu Glu Glu Ala Ile Val Ala Tyr Thr Leu Ala Thr Arg Gly  
 295 300 305  
 Ala Ser Ser Ser Asp Ser Leu Val Ser Pro Pro Glu Ser Pro  
 310 315 320  
 Val Pro Ala Thr Ile Pro Leu Ser Ser Val Ile Val Ala Glu  
 325 330 335  
 Asn Ser Asp Gln Glu Glu Ser Glu Gln Ser Asp Glu Glu Glu  
 340 345 350  
 Glu Glu Gly Ala Gln Glu Glu Arg Glu Asp Thr Val Ser Val  
 355 360  
 Lys Ser Glu Pro Val Ser Glu Ile Glu Glu Val Ala Pro Glu  
 365 370 375  
 Glu Glu Glu Asp Gly Ala Glu Glu Pro Thr Ala Ser Gly Gly  
 380 385 390  
 Lys Ser Thr His Pro Met Val Thr Arg Ser Lys Ala Asp Gln  
 395 400 405

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: cDNA

24

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTATCCTCCT CTAGAATGAA ACAGATTAAG

30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATATATATAT TCTAGAGTTT ACTGGTCGAC

30

## WHAT IS CLAIMED IS:

1. A non-defective recombinant adenovirus comprising a cytomegalovirus gene encoding an immediate early antigen exon-4 subunit protein from a human strain of cytomegalovirus, said gene being under the control of an expression control sequence, said virus being a type 5 strain adenovirus capable of expressing said subunit protein in vivo in an animal.
2. The non-defective recombinant adenovirus according to claim 1, wherein the human cytomegalovirus strain is Towne.
3. An immunogenic composition comprising a non-defective recombinant adenovirus comprising a cytomegalovirus gene encoding an immediate early antigen exon-4 subunit protein from a human strain of cytomegalovirus, said gene being under the control of an expression control sequence, said virus being a type 5 strain adenovirus capable of expressing said subunit protein in vivo in an animal, in a suitable pharmaceutical carrier.
4. The composition according to claim 3, wherein the human cytomegalovirus is Towne strain.
5. A human cytomegalovirus immediate early exon-4 subunit protein produced by an adenovirus expression vector.

6. The use of a non-defective recombinant adenovirus comprising a cytomegalovirus gene encoding an immediate early antigen exon-4 subunit protein from a human strain of cytomegalovirus, said gene being under the control of an expression control sequence, said virus being a type 5 strain adenovirus capable of expressing said subunit protein in vivo in an animal, in admixture with a suitable pharmaceutical carrier in the manufacture of a vaccine composition against cytomegalovirus infection.

7. The use according to claim 6 wherein said vaccine is manufactured for oral administration.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/07107

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : Please See Extra Sheet.

US CL : 424/88, 89; 435/235.1, 69.3, 69.1, 5, 172.3; 436/546; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 89; 435/235.1, 69.3, 69.1, 5, 172.3; 436/546; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

INTELLIGENETICS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,920,209 (DAVIS ET AL.) 24 April 1990, See entire document.	1-7
Y	The EMBO Journal, Volume 5, Number 11, issued November 1986, M.P. Cranage et al., "Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus", pages 3057-3063, See entire document.	1-7
Y	Journal of Virology, Volume 65, Number 9, issued September 1991, N.J. Alp et al., "Fine Specificity of Cellular Immune Responses in Humans to Human cytomegalovirus Immediate-Early 1 Protein", pages 4812-4820, see entire document.	1-7

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"G"	document member of the same patent family

Date of the actual completion of the international search

03 JUNE 1994

Date of mailing of the international search report

JUN 24 1994

 Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

LAURIE SCHEINER

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/ISA/210

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A61K 31/70, 39/12; C12N 7/00, 15/00; C12P 21/06; C12Q 1/70; G01N 33/531